METHODS AND COMPOSITIONS FOR THE ENHANCEMENT OF WOUND HEALING

The present invention claims priority to U.S. Provisional Patent Application Serial Number 60/396,366, filed July 17, 2002, the disclosure of which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

The present invention relates to methods and compositions for wound healing, and in particular, methods and compositions to promote and enhance wound healing.

BACKGROUND

The primary goal in the treatment of wounds is to achieve wound closure. Open cutaneous wounds represent one major category of wounds and include burn wounds, neuropathic ulcers, pressure sores, venous stasis ulcers, and diabetic ulcers. Open cutaneous wounds routinely heal by a process which comprises six major components: i) inflammation, ii) fibroblast proliferation, iii) blood vessel proliferation, iv) connective tissue synthesis v) epithelialization, and vi) wound contraction. Wound healing is impaired when these components, either individually or as a whole, do not function properly. Numerous factors can affect wound healing, including malnutrition, infection, pharmacological agents (*e.g.*, actinomycin and steroids), diabetes, and advanced age [*see* Hunt and Goodson in *Current Surgical Diagnosis & Treatment* (Way; Appleton & Lange), pp. 86-98 (1988)].

With respect to diabetes, it is known that delayed wound healing causes substantial morbidity in patients with diabetes. Diabetes mellitus is a chronic disorder of glucose metabolism and homeostasis that damages many organs. It is the eighth leading cause of death in the United States (Harris *et al.*, *Diabetes* 36:523 (1987)). In persons with diabetes, vascular disease, neuropathy, infections, and recurrent trauma predispose the extremities, especially the foot, to pathologic changes. These pathological changes can ultimately lead to chronic ulceration, which may necessitate amputation.

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The most commonly used conventional modality to assist in wound healing involves the use of wound dressings. In the 1960s, a major breakthrough in wound care occurred when it was discovered that wound healing with a moist occlusive dressings was, generally speaking, more effective than the use of dry, non-occlusive dressings

5   [Winter, Nature 193:293-94 (1962)]. Today, numerous types of dressings are routinely used, including films (*e.g.*, polyurethane films), hydrocolloids (hydrophilic colloidal particles bound to polyurethane foam), hydrogels (cross-linked polymers containing about at least 60% water), foams (hydrophilic or hydrophobic), calcium alginates (nonwoven composites of fibers from calcium alginate), and cellophane (cellulose with a

10   plasticizer) [Kannon and Garrett, Dermatol. Surg. 21:583-590 (1995); Davies, *Burns* 10:94 (1983)]. Unfortunately, certain types of wounds (*e.g.*, diabetic ulcers, pressure sores) and the wounds of certain subjects (*e.g.*, recipients of exogenous corticosteroids) do not heal in a timely manner (or at all) with the use of such dressings.

Several pharmaceutical modalities have also been utilized in an attempt to

15   improve wound healing. For example, treatment regimens involving zinc sulfate have been utilized by some practitioners. However, the efficacy of these regimens has been primarily attributed to their reversal of the effects of sub-normal serum zinc levels (*e.g.*, decreased host resistance and altered intracellular bactericidal activity) [Riley, *Am. Fam. Physician* 24:107 (1981)]. While other vitamin and mineral deficiencies have also been

20   associated with decreased wound healing (*e.g.*, deficiencies of vitamins A, C and D; and calcium, magnesium, copper, and iron), there is no strong evidence that increasing the serum levels of these substances above their normal levels actually enhances wound healing. Thus, except in very limited circumstances, the promotion of wound healing with these agents has met with little success.

25   What is needed is a safe, effective, and interactive means for enhancing the healing of wounds of all types and without regard to the type of wound or the nature of the patient population to which the subject belongs.

SUMMARY OF THE INVENTION

30   The present invention relates to methods and compositions for wound healing, and in particular, methods and compositions to promote and enhance wound healing. For

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example, in some embodiments, the present invention provides a method for treating a wound, comprising providing an MMP prodomain peptide, and a subject having at least one wound; and administering the peptide to the subject under conditions such that the healing of the wound is promoted. In some embodiments, the subject is a patient with

5 diabetes. In other embodiments, the subject is a burn patient. In still further embodiments, the wound is a chronic wound. In some embodiments, the peptide is derived from MMP 1 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDVAQF-NH$_2$). In other embodiments, the peptide is derived from MMP 7 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-

10 PRCGVPDVAEY-NH$_2$). In still further embodiments, the peptide is derived from MMP 2 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGNPDVANY-NH$_2$). In yet other embodiments, the peptide is derived from MMP 9 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDLGRFQ-NH$_2$).

15 The present invention further provides a kit for the treatment of wounds in a subject, comprising an MMP prodomain peptide; and instructions for using the peptide to treat wounds in the subject. In some embodiments, the subject is a patient with diabetes. In other embodiments, the subject is a burn patient. In still further embodiments, the wound is a chronic wound. In some embodiments, the peptide is derived from MMP 1

20 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDVAQF-NH$_2$). In other embodiments, the peptide is derived from MMP 7 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDVAEY-NH$_2$). In still further embodiments, the peptide is derived from MMP 2 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-

25 PRCGNPDVANY-NH$_2$). In yet other embodiments, the peptide is derived from MMP 9 (*e.g.*, including, but not limited to, a peptide with the amino acid sequence Ac-PRCGVPDLGRFQ-NH$_2$).

DESCRIPTION OF THE FIGURES

30 Figure 1 shows induction of human microvascular cell invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$. X axis, log of the peptide concentration in

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ng/ml. The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Y axis, percent of microvascular cells invaded. Means are shown with first standard deviations.

Figure 2 shows inhibition of Ac-PRCGVPDVAEY-NH$_2$ peptide–induced microvascular cell invasion by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. X axis, media constituents: PRC, 600 ng/ml Ac-PRCGVPDVAEY-NH$_2$; COMY4A2, 300 µg/ml anti MMP 1 monoclonal antibody; IgG, 300 µg/ml IgG. Y axis, percentage of invaded microvascular cells relative to the percentage invaded in the presence of 600 ng/ml Ac-PRCGVPDVAEY-NH$_2$ peptide without blocking antibody. Means and first standard deviations are shown.

Figure 3 shows induction of human neonatal fibroblast invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$. X axis, log of the peptide concentration in ng/ml. The peptide used is the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$. The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Y axis, percent of fibroblasts invaded. Means are shown with first standard deviations.

Figure 4 shows inhibition of Ac-PRCGVPDVAEY-NH$_2$ peptide–induced fibroblast invasion by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. X axis, media constitutents: PRC, 600 ng/ml Ac-PRCGVPDVAEY-NH$_2$; COMY4A2, 300 µg/ml anti MMP 1 monoclonal antibody; IgG, 300 µg/ml IgG. Y axis, percentage of invaded fibroblasts relative to the percentage invaded in the presence of 600 ng/ml Ac-PRCGVPDVAEY-NH$_2$ peptide without blocking antibody. Means and first standard deviations are shown.

Figure 5 shows induction of human keratinocyte invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$. X axis, log of the peptide concentration in ng/ml. The peptide used is the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$. The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Y axis, percent of keratinocytes invaded. Means are shown with first standard deviations.

Figure 6 shows inhibition of Ac-PRCGVPDVAEY-NH$_2$ peptide–induced keratinocyte invasion by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. X axis, media constitutents: PRC, 600 ng/ml Ac-PRCGVPDVAEY-NH$_2$; COMY4A2, 300 µg/ml anti MMP 1 monoclonal antibody; IgG, 300 µg/ml IgG. Y axis, percentage of invaded keratinocytes relative to the percentage invaded in the

presence of 600 ng/ml Ac-PRCGVPDVAEY-NH$_2$ peptide without blocking antibody. Means and first standard deviations are shown.

Figure 7 shows enhancement of C57BL6Ks*db/db* obese diabetic mouse wound healing by a topical application of the MMP 2 switch sequence. X axis, days since wounding. Y axis, wound area, relative to the initial wound area (13 to 19 mm^2). Db/db: healing–impaired, homozygous *db/db*, obese diabetic mice; db/+: normally healing, heterozygous *db/+*, non–diabetic littermates. Mean wound areas are shown.

GENERAL DESCRIPTION OF THE INVENTION

Patients with diabetes exhibit delayed wound healing because of changes in vascular basement membranes and extracellular matrix that are believed to delay the entry of cells into wounds (Williamson *et al.*, (1988) Diab. Metabol. Rev. 4: 339-370). As a result of impaired wound healing, diabetics often encounter considerable difficulties in healing ulcers and controlling infection, especially in the lower extremities. A portion of these slowly–healing wounds can become chronic, and necessitate amputation (Pierce, (2001) Am. J. Pathol. 159: 399-403). Topical agents that can induce the invasion of wound provisional matrix by surrounding fibroblasts and microvascular cells, and accelerate closure by stimulating epithelial cell migration across the provisional matrix surface could have significant therapeutic value in speeding wound healing, reducing the chances of infection, and thereby preventing the development of chronic, non–healing wounds, whether used alone or in combination with agents to control infection. Although not curative of the diabetes, these topical agents would thus make it much easier for patients to live with their disease.

The C57BL6Ks *db/db* mouse has been used extensively as a model of diabetic wounds in humans (Coleman, (1982) Diabetes 31: 1-6.). This mouse strain has a leptin receptor deficiency caused by a specific point mutation that affects splicing (Lee *et al.*, (1996) Nature 379: 632-635). *db/db* mouse wounds have been shown to have a number of molecular features in common with human diabetic wounds. For example, the wounds of *db/db* mice exhibit a decreased influx of inflammatory cells, as well as reduced growth factor expression (Fahey *et al.*, (1991) J. Surg. Res. 50: 308-313; Werner *et al.*, (1994) J. Invest. Dermatol. 103: 469-473). The demonstration that impaired healing in *db/db* mice

could be reversed by the topical application of polypeptide growth factors, including fibroblast growth factor–2 (FGF-2) and platelet–derived growth factor–BB (PDGF-BB) (Tsuboi et al., (1990) J. Exp. Med. 172: 245-251), lead to the successful trial of platelet derived growth factor–BB in human diabetics (Smiell et al., (1999) Wound Repair

5   Regen. 7: 335-346). Also, the PHSRN sequence of the fibronectin cell–binding domain has been shown to induce $\alpha5\beta1$ integrin fibronectin receptor–mediated fibroblast and keratinocyte invasion of the extracellular matrix in vitro, as well as stimulate the rapid reepithelialization and contraction of dermal wounds in db/db mice, when applied as a peptide, Ac-PHSRN-NH$_2$ (Livant et al., (2000) J. Clin. Invest. 105: 1537-1545). The

10   successful completion of reepithelialization in this impaired wound healing model has been interpreted as indicating avoidance of the destructive matrix metalloproteinase expression profile that limits closure and can result in chronic, non–healing wounds (Neely et al., (2000) J. Burn Care & Rehab. 21: 395-402). Thus, many investigators have found the C57BL6Ksdb/db mouse strain to be a very useful model for evaluating the

15   effects of topically added polypeptides on healing–impaired wounds, and predicting their effects in the wounds of human diabetics.

      Matrix metalloproteinases are a large family of zinc proteinases that are secreted by both resident and inflammatory cells. Collectively, they are capable of remodeling or degrading virtually all of the molecules of the extracellular matrix. This processing of

20   the extracellular matrix is crucial for wound healing and angiogenesis, as well as for development, differentiation, cell migration, and tumor cell metastasis. Interstitial collagenase, or MMP–1, is very important in wound healing because this metalloproteinase has been shown to play important roles in reepithelialization, formation of the provisional matrix, and angiogenesis. The triple helical structure of fibrillar

25   collagen makes it very resistant to proteolysis, and only a very limited number of MMPs, including MMP–1 or interstitial collagenase, can cleave it (Stricker et al., (2001) J. Biol. Chem. 276: 29375-29381).

      Many experimental findings indicate the importance of MMP–1 activity in reepithelialization. MMP–1 is expressed by keratinocytes migrating on collagen (Pilcher

30   et al., (1997) J. Cell Biol. 137: 1445-1457). Its accumulation has been shown to be stimulated by the interaction of the invasion–inducing, PHSRN sequence of the

fibronectin cell–binding domain with the $\alpha5\beta1$ integrin fibronectin receptor in normal epithelial cells and in their metastatic counterparts, as well as in fibroblasts (Livant *et al.*, (2000) Cancer Res. 60: 309-320; Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). After secretion, MMP–1 has been shown to interact with the surface $\alpha2\beta1$ integrin of

5   keratinocytes during their migration on type I collagen (Dumin *et al.*, (2001) J. Biol. Chem. 276: 29368-29374). Also, MMP–1 has been shown to be substantially upregulated in motile keratinocytes at the leading edge of the new epithelium of wounds, and to be required for successful reepithelialization (Saarialho–Kere *et al.*, (1995) J. Invest. Dermatol. 104: 982-988; Agren *et al.*, Exp. Derm. 10: 337-348). Total MMP–1

10   activity has been shown to increase approximately 100–fold in the wounded skin of healthy human volunteers, relative to unwounded skin (Petri *et al.*, (1997) Exp. Dermatol. 6: 133-139).

MMP–1 expression is also important in the formation of granulation tissue. Cultured granulation tissue fibroblasts from human volunteers have been found to

15   expressed greatly increased levels of MMP–1 mRNA (Petri *et al.*, (1997) Exp. Dermatol. 6: 133-139). MMP –1 transcription has been found to be upregulated in human dermal fibroblasts in response to wounding (Abe *et al.*, (2001) Eur. J. Dermatol. 11: 112-116). Also, PDGF–BB, a growth factor that stimulates dermal wound healing in both humans and *db/db* mice (Tsuboi, R. and Rifkin, D. B. (1990) J. Exp. Med. 172: 245-251; Smiell

20   *et al.*, (1999) Wound Repair Regen. 7: 335-346), has been found to consistently up regulate MMP–1 expression in human fibroblasts (Tan *et al.*, (1995) Biochem. J. 310: 585-588).

Many studies also indicate that MMP–1 activity is important in angiogenesis, both *in vitro* and *in vivo*. For example, MMP–1 has been shown to be required for

25   angiogenesis by human umbilical vein endothelial cells in collagen gels (Fisher et al. (1994) Dev. Biol. 162: 499-510). Also, microvascular endothelial cells overexpressing FGF–1 have been shown to have a greatly enhanced migration rate through collagen I matrices that depends on MMP–1 activity (Partridge et al., (2000) J. Cell. Biochem. 78: 487-499). Immunofluorescent staining has also shown that MMP–1 is upregulated in

30   early microvessels, as well as in microvessels involved in elongation and spout formation, in cultured human fetal skin (Karelina *et al.*, (1995) J. Invest. Dermatol. 105:

411-417). In addition, deficient MMP-1 activation has been shown to contribute significantly to aging–impaired angiogenesis in humans (Reed *et al.*, (2000) J. Cell Biochem. 77: 116-126).

Thus, the induction and activation of metalloproteinases, in particular MMP-1, are very important processes for wound reepithelialization, provisional matrix formation and maturation, and angiogenesis. The development of topical agents to stimulate these processes is very useful in promoting wound healing and preventing the development of non–healing wounds in diabetic patients. In some embodiments, based on the importance of MMP –1 activity in reepithelialization, provisional matrix formation, and angiogenesis, the present invention provides peptide agents that promote the activation of MMP-1 in fibroblasts and endothelial cells, as well as in keratinocytes, to promote the closure of dermal wounds in obese diabetic mice. Experiments conducted during the course of development of the present invention demonstrated that peptides containing the highly conserved MMP prodomain cysteine switch region are potent inducers of invasion, that this invasion depends specifically on MMP-1 activity, and that it is not α5β1–mediated. While the present invention is not limited to any mechanism and an understanding of the mechanism is not necessary to practice the present invention, it is contemplated that the effect is the result of a pathway independent of PHSRN–induced invasion since the PHSRN sequence has been shown to interact only with α5β1 (Aota, S., Nagai, T., and Yamada, K. M. (1991) Biol. Chem. 266: 15938-15943). Further experiments demonstrated that a single topical application of a peptide containing the MMP prodomain cysteine switch region enhances *db/db* wound healing, making wound contraction and closure at least as rapid as that observed in untreated, normally healing, *db/+* littermates.

It has long been known that MMPs are expressed as latent proenzymes, which must be activated by proteolytic cleavage of the prodomain, and that a highly conserved cysteine at a constant position in the prodomain, called the cysteine switch, functions in activation. This cysteine has been shown to coordinate with a zinc cation at the active site, thereby preventing hydration of the cation and subsequent proteolytic activation (Springman *et al.*, (1990) Proc. Natl. Acad. Sci. 87: 364-368; Morgunova *et al.*, (1999) Science 284: 1667-1669). Latent forms of MMPs can be activated by a variety of

treatments affecting the cysteine switch (Springman *et al.*, (1990) Proc. Natl. Acad. Sci. 87: 364-368; Morgunova *et al.*, (1999) Science 284: 1667-1669). Experiments conducted during the course of development of the present invention demonstrated that exposure to the conserved cysteine switch region of the cleaved propeptide can activate MMP 1 to

5   induce invasion and enhance diabetic wound healing.

DEFINITIONS

     To facilitate understanding of the invention set forth in the disclosure that follows, a number of terms are defined below.

10   The term "wound" refers broadly to injuries to the skin and subcutaneous tissue initiated in different ways (*e.g.*, pressure sores from extended bed rest and wounds induced by trauma) and with varying characteristics. Wounds may be classified into one of four grades depending on the depth of the wound: i) Grade I: wounds limited to the epithelium; ii) Grade II: wounds extending into the dermis; iii) Grade III: wounds

15   extending into the subcutaneous tissue; and iv) Grade IV (or full-thickness wounds): wounds wherein bones are exposed (*e.g.*, a bony pressure point such as the greater trochanter or the sacrum). The term "partial thickness wound" refers to wounds that encompass Grades I-III; examples of partial thickness wounds include burn wounds, pressure sores, venous stasis ulcers, and diabetic ulcers. The term "deep wound" is meant

20   to include both Grade III and Grade IV wounds. The present invention contemplates treating all wound types, including deep wounds and chronic wounds.

     The term "chronic wound" refers to a wound that has not healed within 30 days.

     The phrases "promote wound healing," "enhance wound healing," and the like refer to either the induction of the formation of granulation tissue of wound contraction

25   and/or the induction of epithelialization (*i.e.*, the generation of new cells in the epithelium). Wound healing is conveniently measured by decreasing wound area.

     The phrase "wound fluid contents" refers to liquid associated with a wound, as well as cells, cell factors, ions, macromolecules and protein material suspended such liquid at the wound site.

30   The term "subject" refers to both humans and animals.

The terms "enclosure," "compartment," and the like refer broadly to any container capable of confining a solid support within a defined location.

The term "solid support" refers broadly to any support, including, but not limited to, microcarrier beads, gels, BAND-AIDS and dressings.

5   The term "dressing" refers broadly to any material applied to a wound for protection, absorbance, drainage, etc. Thus, adsorbent and absorbent materials are specifically contemplated as a solid support. Numerous types of dressings are commercially available, including films (*e.g.*, polyurethane films), hydrocolloids (hydrophilic colloidal particles bound to polyurethane foam), hydrogels (cross-linked

10   polymers containing about at least 60% water), foams (hydrophilic or hydrophobic), calcium alginates (nonwoven composites of fibers from calcium alginate), and cellophane (cellulose with a plasticizer) [Kannon and Garrett, *Dermatol. Surg.* 21:583-590 (1995); Davies, *Burns* 10:94 (1983)]. The present invention specifically contemplates the use of dressings impregnated with the wound healing promoting and enhancing compounds of

15   the present invention.

The term "biocompatible" means that there is minimal (*i.e.*, no significant difference is seen compared to a control), if any, effect on the surroundings. For example, in some embodiments of the present invention, the dressing comprises a biocompatible membrane.

20   The term "peptide derivative" refers to compound having an imino group (-NH-), and more particularly, a peptide bond. Peptides may be regarded as substituted amides. Like the amide group, the peptide bond shows a high degree of resonance stabilization. The C-N single bond in the peptide linkage has typically about 40 percent double-bond character and the C=O double bond about 40 percent single-bond character.

25   "Protecting groups" are those groups that prevent undesirable reactions (such as proteolysis) involving unprotected functional groups. In one embodiment, the present invention contemplates that the protecting group is an acyl or an amide. In one embodiment, the acyl is acetate. In another embodiment, the protecting group is a benzyl group. In another embodiment, the protecting group is a benzoyl group. The present

30   invention also contemplates combinations of such protecting groups.

DETAILED DESCRIPTION OF THE INVENTION

Rapid induction of fibroblast, keratinocyte, and endothelial cell migration into dermal wounds is crucial for successful tissue repair. Interstitial collagenase or MMP–1 is a key feature of healing wounds because it has been shown to function in reepithelialization, formation of the provisional matrix, and angiogenesis. Active MMP–1 is associated with keratinocytes, as they migrate across the surface of the provisional matrix. It is also associated with fibroblasts during invasion of the provisional matrix. Finally, it is associated with the invading sprouts of capillaries as they invade the extracellular matrix of the wound during angiogenesis. MMPs are expressed as latent proenzymes, that must be activated by proteolytic cleavage of their prodomains. A highly conserved cysteine, corresponding to C^{73} of MMP–1, is found at a constant position in the prodomain, functions in activation. This cysteine residue is called the cysteine switch because it coordinates with the zinc cation of the active site in the proprotein, thus preventing the hydration of the zinc cation and blocking the active site. During MMP activation, cleavage of the prodomain removes the cysteine switch, and allows hydration of the active site zinc cation, thus making the active site accessible to substrate collagen.

A method for activating the cysteine switch of MMP–1 is needed. The present invention is not limited to a particular mechanism. Indeed, an understanding of the mechanism is not necessary to practice the present invention. Nonetheless, it is contemplated that, because compounds that can induce the local conversion of proenzyme to active MMP–1 in epithelial cells, fibroblasts and microvascular endothelial cells are contemplated to be potent topical agents for promoting wound healing. These agents are especially useful in diabetic patients because the cellular invasion of diabetic wounds is known to contribute to their impaired healing. In addition to the therapeutic potential of these peptide agents to alleviate some of the worst consequences of diabetic disease, *i.e.*–the development of chronic wounds in the extremities that can lead to amputation, the present invention provides peptides containing the highly conserved cysteine switch regions of MMP prodomains for use as potent stimulators of MMP –1 activity, and thus of extracellular matrix invasion by keratinocytes, fibroblasts, and endothelial cells. The present invention further provides methods of using topical

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application of MMP cysteine switch–containing peptides to cure the diabetic defect in wound closure in *db/db* mice by stimulating reepithelialization, provisional matrix formation, and angiogenesis. Thus, in some embodiments, the present invention provides agents capable of stimulating MMP–1 activation to promote reepithelialization,

5   provisional matrix formation, and angiogenesis.

I.   Peptides

In some embodiments, the present invention provides peptides derived from MMP proteins. The present invention is not limited to peptides derived from a particular

10   MMP protein. Peptides and mimetics (See section II below) derived from a variety of MMP proteins including, but not limited to, MMP 1, MMP 2, MMP 7, and MMP 9 are contemplated. As described above, it is contemplated that peptides comprising cysteine switch sequences are particularly preferred for use in the compositions and methods of the present invention. Exemplary peptide sequences include, but are not limited to, Ac-

15   PRCGVPDVAQF-NH$_2$ (MMP 1 prodomain peptide), Ac-PRCGVPDVAEY-NH$_2$ (MMP 7), Ac-PRCGNPDVANY-NH$_2$ (MMP2), and Ac-PRCGVPDLGRFQ-NH$_2$ (MMP 9).

The present invention also contemplates the use of variants of any of the specified sequences disclosed herein. Variants include those peptide with conserved amino acid change (e.g., substitution of amino acids with similar charge, size, hydophobicity, etc.),

20   peptide containing additional C-terminal or N-terminal amino acids, as well as other functional equivalents. Functional equivalents can be determined by using simple assays, such as those described in Section III, below.

II.   Mimetics

25   Compounds mimicking the necessary conformation for recognition and docking to the receptor binding to the peptides of the present invention are contemplated as within the scope of this invention. For example, mimetics of the peptides described in Section I above are contemplated. A variety of designs for such mimetics are possible. For example, cyclic peptides, in which the necessary conformation for binding is stabilized

30   by nonpeptides, are specifically contemplated. United States Patent No. 5,192,746 to Lobl, *et al*, United States Patent No. 5,169,862 to Burke, Jr., *et al*, United States Patent

No. 5,539,085 to Bischoff, *et al*, United States Patent No. 5,576,423 to Aversa, *et al*, United States Patent No. 5,051,448 to Shashoua, and United States Patent No. 5,559,103 to Gaeta, *et al*, all hereby incorporated by reference, describe multiple methods for creating such compounds.

5   Synthesis of nonpeptide compounds that mimic peptide sequences is also known in the art. Eldred, *et al*, (*J. Med. Chem.* 37:3882 (1994)) describe nonpeptide antagonists that mimic the Arg-Gly-Asp sequence. Likewise, Ku, *et al*, (*J. Med. Chem.* 38:9 (1995)) give further elucidation of the synthesis of a series of such compounds. Such nonpeptide compounds that mimic the peptides of the present invention are specifically contemplated

10 by the present invention.

        The present invention also contemplates synthetic mimicking compounds that are multimeric compounds that repeat the relevant peptide sequence. In one embodiment of the present invention, it is contemplated that the relevant peptide sequence is PRCGV/NPDVA sequence of the MMP prodomain. As is known in the art, peptides can

15 be synthesized by linking an amino group to a carboxyl group that has been activated by reaction with a coupling agent, such as dicyclohexylcarbodiimide (DCC). The attack of a free amino group on the activated carboxyl leads to the formation of a peptide bond and the release of dicyclohexylurea. It can be necessary to protect potentially reactive groups other than the amino and carboxyl groups intended to react. For example, the α-amino

20 group of the component containing the activated carboxyl group can be blocked with a tertbutyloxycarbonyl group. This protecting group can be subsequently removed by exposing the peptide to dilute acid, which leaves peptide bonds intact.

        With this method, peptides can be readily synthesized by a solid phase method by adding amino acids stepwise to a growing peptide chain that is linked to an insoluble

25 matrix, such as polystyrene beads. The carboxyl-terminal amino acid (with an amino protecting group) of the desired peptide sequence is first anchored to the polystyrene beads. The protecting group of the amino acid is then removed. The next amino acid (with the protecting group) is added with the coupling agent. This is followed by a washing cycle. The cycle is repeated as necessary.

30   In one embodiment, the mimetics of the present invention are peptides having sequence homology to the above-described peptides (including, but not limited to,

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peptides in which L-amino acids are replaced by their D-isomers). One common methodology for evaluating sequence homology, and more importantly statistically significant similarities, is to use a Monte Carlo analysis using an algorithm written by Lipman and Pearson to obtain a Z value. According to this analysis, a Z value greater than 6 indicates probable significance, and a Z value greater than 10 is considered to be statistically significant. W.R. Pearson and D.J. Lipman, *Proc. Natl. Acad. Sci.* (USA), 85:2444-2448 (1988); D.J. Lipman and W.R. Pearson, *Science*, 227:1435-1441 (1985). In the present invention, synthetic polypeptides useful in wound healing are those peptides with statistically significant sequence homology and similarity (Z value of Lipman and Pearson algorithm in Monte Carlo analysis exceeding 6).

III.   Assays

In preferred embodiments, candidate peptides and mimetics are screened for their activity in invasion induction, stimulation of activated MMP, and stimulation of wound healing. Any suitable assay may be utilized for such experiments including, but not limited to, those described below.

A.   Invasion Induction

In some embodiments, the present invention provides methods of using MMP mimetics for induction of invasion. In some embodiments, candidate peptides of the present invention are assayed for their ability to induce invasion. In some embodiments, invasion induction is quantitated by exposing normal human keratinocytes, fibroblasts, and microvascular cells to peptides, and placed on SU-ECM *in vitro* invasion substrates. The role of activated MMPs in invasion induction is assessed functionally through the use of the blocking anti–MMP 1 COMY 4A2 antibody (Birkedal-Hansen *et al.*, Biochemistry 27: 6751-6758) with appropriate isotype controls. Blocking anti–MMP 2, and –9 antibodies (Fridman *et al.*, (1992) J. Biol. Chem. 267(22):15398-15405; Schnaper *et al.*, (1993) J. Cell. Physiol. 156(2): 235-246.) are also utilized to ascertain if MMP 2 and MMP 9 are also involved in switch peptide–induced invasion.

B. Activation of MMP

Peptides are screened for their ability to stimulate the production of activated MMP (*e.g.*, MMP 1), specifically by affecting the interactions of the cysteine switch of the MMP 1 N–terminal propeptide domain with the active site, peptides in which the

5 cysteine switch amino acid is replaced by serine, will be synthesized. The C–to –S point mutation at this location has been shown to inactivate the cysteine switch in the MMP 1 proprotein (Windsor *et al.*, (1991) Biochem. 30: 641-647). The lack of ability of the C to S peptide to induce invasion of SU-ECM by keratinocytes, fibroblasts, and endothelial cells is verified using SU-ECM *in vitro* invasion assays, in parallel with assays

10 employing the parental peptide. The role of activated MMP 1 in invasion is assessed functionally on SU-ECM invasion substrates (Livant *et al*, (1995) *Cancer Res.* 55: 5085-5093) through the use of the blocking anti–MMP 1 COMY 4A2 antibody (Birkedal-Hansen *et al.*, Biochemistry 27: 6751-6758) with appropriate isotype controls. MMP 1 activity is also be quantitated by zymographic analysis of the media from adherent

15 keratinocytes, fibroblasts, and endothelial cells exposed to either the C to S substituted or non-substituted peptides, as described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545,11). Similar experiments are performed for the switch region peptide from the MMP 7 (Gaire *et al.*, (1994) J. Biol. Chem. 269: 2032-2040), MMP 2 (Huhtala *et al.*, (1990) J. Biol. Chem. 265: 11077-11082), and MMP 9 (Wilhelm *et al.*, (1989) J. Biol.

20 Chem. 264: 17213-17221) prodomains.

C. Wound Healing

Peptides are further evaluated for their ability to stimulate the diabetic wound healing. The peptides are topically applied to duplicate dermal wounds in healing–

25 impaired obese diabetic mice (Greenhalgh *et al.*, (1990) Am. J. Pathol. 136: 1235-1246), according to published protocols (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Wound closure times are determined in both treated and untreated mice. To confirm the importance of the cysteine switch residue, some wounds are treated with the C to S substituted peptides. To quantitate the extent of reepithelialization for several days

30 wounding, wounds are excised, sectioned, stained, and examined microscopically. To evaluate the provisional matrix for indications of wound contraction and angiogenesis,

the presence of activated fibroblasts, collagenous fibers, microvasculature, and wound macrophages is assessed in sectioned and stained wounds for several days following wounding as described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). These assays are performed using peptides derived from MMP 1, MMP 2, MMP 7, and MMP 9.

5

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

10   In the experimental disclosure which follows, the following abbreviations apply: N (normal); M (molar); mM (millimolar); μM (micromolar); mol (moles); mmol (millimoles); μmol (micromoles); nmol (nanomoles); pmol (picomoles); g (grams); mg (milligrams); μg (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μl (microliters); cm (centimeters); mm (millimeters); μm (micrometers);

15   nm (nanometers); DS (dextran sulfate); °C (degrees Centigrade); and Sigma (Sigma Chemical Co., St. Louis, MO).

Example 1

The Effect of MMP peptides on in vitro Invasion Induction

20

A.   **Materials and Methods**

About 1×10^5 human microvascular cells, neonatal fibroblasts, or undifferentiated human keratinocytes were suspended in medium containing 5% fetal calf serum (FCS), mixed with varying concentrations of the MMP 7 switch peptide, Ac-PRCGVPDVAEY-

25   NH$_2$ (Gaire *et al.*, (1994) J. Biol. Chem. 269: 2032-2040), function–blocking anti human MMP 1 monoclonal antibody (Birkedal-Hansen *et al.*, (1988) Biochemistry 27; 6751-6758), or total human IgG, and placed on the surfaces of SU-ECM *in vitro* invasion substrates. SU-ECM invasion substrates are made as described from sea urchin embryos, and contain a blastocoelic cavity enclosed by an intact basement membrane and

30   associated extracellular matrix (Livant *et al.*, (1995) Cancer Research 55, 5085-5093). After 4 hours of incubation (the time necessary to observe maximal invasion

percentages), the invasion percentage in each assay was determined by microscopic examination of the SU-ECM invasion substrates and their associated cells at 400–fold magnification. Invasion percentages are the ratios of the numbers of cells located on the internal surfaces of SU-ECM to the total numbers of cells in contact with either the

5   internal or the external surfaces of SU-ECM, and multiplied by 100.

B.   Results

The results are shown in Figures 1-6. Figure 1 shows induction of human microvascular cell invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$.

10   The peptide used is the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$. The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Figure 2 shows that Ac-PRCGVPDVAEY-NH$_2$ peptide–induced microvascular cell invasion is inhibited by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. Figure 3 shows that invasion of human neonatal fibroblasts is enhanced by the MMP 7

15   switch peptide, Ac-PRCGVPDVAEY-NH$_2$. Figure 4 shows that Ac-PRCGVPDVAEY-NH$_2$ peptide–induced fibroblast invasion is inhibited by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. Figure 5 shows the induction of human keratinocyte invasion by the MMP 7 switch peptide, Ac-PRCGVPDVAEY-NH$_2$. The peptide used is the MMP 7 switch peptide, Ac-

20   PRCGVPDVAEY-NH$_2$. The peptide concentrations used are as follows: 2, 6, 20, and 60 ng/ml. Figure 6 shows that Ac-PRCGVPDVAEY-NH$_2$ peptide–induced keratinocyte invasion is inhibited by blocking anti MMP 1 COMY4A2 monoclonal antibody, but not by total serum IgG. In conclusion, the MMP 7 switch peptide induces dose-dependent invasion by human fibroblasts, keratinocytes, and microvascular cells. The inhibition

25   was blocked by a MMP 1–specific monoclonal antibody, but not by serum IgG.

30

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Example 2

Enhancement of Wound Healing in Diabetic Mice

A.   Methods

Each treatment group consisted of 10 mice with duplicate, dermal wounds of 12.5 to 16.5 mm^2 made in the shoulder area. Complete methods were as described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Treated animal were treated with 10 μg switch (PRC) sequence from MMP2 (Ac-PRCGNPDVANY-NH$_2$). Wound areas were measured by integration of digital images.

B.   Results

The results are shown in Figure 7. One treatment with the PRC peptide just after wounding decreased the time required for closure of all db/db wound by 50%, relative to untreated db/db mice. The closure of PRC-treated wounds appears to be as rapid as that of db/+ non-diabetic mice.

Example 3

Methods

This example described methods useful in performing some embodiments of the present invention.

Cell culture and SU-ECM invasion assays. In SU-ECM *in vitro* invasion substrates, a basement membrane surrounds a blastocoel, in which invading cells localize shortly after suspension and placement on the outer surfaces. Even in the presence of serum, these invasion substrates have been shown to be free of background invasion by normal cells (Livant *et al.*, (1995) *Cancer Res.* 55: 5085-5093; Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545; Livant *et al.*, (2000) Cancer Res. 60: 309-320). These invasion substrates have been used to define the invasion–promoting activity of the PHSRN sequence from the fibronectin cell–binding domain, a previously unanticipated function of this sequence. Ac-PHSRN-NH$_2$ peptide has been shown to be a potent topical agent for stimulating dermal wound healing in obese diabetic C57BL6Ks*db/db* mice (Livant *et*

al., (2000) J. Clin. Invest. 105: 1537-1545). Because they are naturally serum–free, the SU-ECM invasion substrates have been used to define a potent inhibitor of plasma fibronectin–induced invasion by metastatic prostate cancer cells; and this inhibitor has been shown to be an effective and non–toxic anti-tumorigenic and anti-metastatic agent

5   in Copenhagen rats (Livant *et al.* (2000) Cancer Res. 60: 309-320), as well as in nude mice bearing human prostate cancer tumors (Windsor *et al.*, (1991) Biochem. 30: 641-647; 38).

    In vitro invasion assays are performed using SU-ECM with normal human fibroblasts, keratinocytes, and endothelial cells as previously described (Livant *et al.*,

10   (2000) J. Clin. Invest. 105: 1537-1545). Undifferentiated, first or second passage human keratinocytes are obtained from the laboratory of C. Marcelo at the University of Michigan. Keratinocytes are cultured in serum-free keratinocyte growth medium (Clonetics, San Diego CA). Neonatal fibroblasts are obtained from the laboratory of J. Varani at the University of Michigan. First or second passage neonatal fibroblasts are

15   cultured as previously described (Livant *et al.*,(1995) *Cancer Res.* 55: 5085-5093). Normal human endothelial cells (microvascular cells) are obtained from the laboratory of D. Arenberg at the University of Michigan. Microvascular cells are cultured as described (White *et al.*, (2001) J. Immunol. 16: 5362-5366). Cells from single cell suspensions (made with 0.25% trypsin/EDTA from Gibco Life Technologies, Grand Island NY) are

20   rinsed, and briefly prebound to the following peptides (1, 3, 4, 5): Ac-PRCGVPDVAQF-NH_2 (from MMP–1), Ac-PRCGVPDVAEY-NH_2 (from MMP–7), Ac-PRCGNPDVANY-NH_2 (from MMP–2), Ac-PRCGVPDLGRFQ-NH_2 (from MMP–9). The testing of the peptides containing the C–to–S substitution is used to determine if invasion–promoting activities result from their interactions with the cysteine switch of inactive MMP–1. Thus,

25   the effects on invasion of the corresponding 4 peptides containing the C–to–S substitution at the third position: Ac-PRSGVPDVAQF-NH_2, Ac-PRSGVPDVAEY-NH_2, Ac-PRSGNPDVANY-NH_2, and Ac-PRSGVPDLGRFQ-NH_2 is also assayed. To verify the lack of activity in the peptides containing the C–to–S substitution at the third position, peptides consisting of the arbitrarily scrambled sequences from the MMP–1, –7, –2, and

30   –9 switch region propeptides listed above are also tested. Final peptide concentrations in the SU-ECM invasion assays will range from 2 to 60 ng per ml. These concentrations are

derived from the dose response curve shown in Figure 1 of Example 1. After prebinding

to each of the peptides listed above, fibroblasts, endothelial cells, and keratinocytes are

placed on SU-ECM invasion substrates in the appropriate media for 4 hours at 37°C, the

time required to observe maximal invasion percentages (Livant *et al.*, (1995) *Cancer Res.*

5   *55*: 5085-5093). Cellular viability in SU-ECM invasion assays are next determined, and

are expected to range from 90% to 99% (Livant *et al.*, (1995) *Cancer Res. 55*: 5085-

5093). Invasion percentages are the ratio of the number of cells located in the

blastocoelic cavities of SU-ECM invasion substrates to the total number of single cells

adhering to them on their exterior and their interior surfaces. Each invasion percentage is

10   the result of 3 to 4 independent determinations involving the scoring of the positions of

all individual cells adhering to SU-ECM, typically about 100 individual cells for each

determination.

Peptide synthesis. Peptides are synthesized using standard Fmoc/t-butyl

15   protection strategies at 25 to 100 micromole scales on a Ranin Symphony multiple

peptide synthesizer at the University of Michigan as previously described (Livant *et al.*,

(2000) J. Clin. Invest. 105: 1537-1545). The synthetic methods employed are a

modification of the solid-phase method of Merrifield. Peptides are synthesized on a

chlorotrityl resin and C-terminally amidated peptides are synthesized on Rink resin. The

20   completed peptides are cleaved from the resin support and the side chain protecting

groups simultaneously removed by anhydrous trifluoroacetic acid (TFA). The peptides

are then precipitated with diethylether, purified by preparative HPLC, and lyophilized.

Peptide purities are assessed by reverse phase HPLC, performed according to standard

procedures, as previously described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-

25   1545). Peptide structures are then confirmed by mass spectrometry and amino acid

analysis as described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Because the

presence of TFA, a very strong acid, could affect peptide activity in aqueous solution or

injure the mice, residual TFA is removed by gel permeation chromatography as

previously described (Schnaper *et al.*, (1993) J. Cell. Physiol. 156(2): 235-246).

30   Peptides are then lyophilized to remove the acetic acid and stored as lyophilized solids at

–20°C until solubolization in normal saline at concentrations of 1 to 5 mg per ml for use *in vitro* invasion assays, or as topical agents in *db/db* mouse wounds.

Use of function–blocking antibodies in invasion assays The effects of function–
5   blocking anti MMP 1 (COMY 4A2) antibody on Ac-PRCGVPDVAQF-NH$_2$ (MMP–1 switch peptide)–induced invasion is determined for keratinocytes, fibroblasts, and microvascular cells, placed on SU-ECM *in vitro* invasion substrates. To assess the specificity of the role of MMP–1 in switch peptide–induced invasion, the effects of function–blocking anti MMP 2 (CA 4001), and MMP 9 (GE 213) monoclonal antibodies

10   (Schnaper *et al.*, (1993) J. Cell. Physiol. 156(2): 235-246) are also determined. All of the antibodies are purchased from Chemicon International (Temecula CA). Antibodies are prebound to cells for 30 minutes on ice in concentrations ranging from 10 to 300 μg per ml, prior to their placement on invasion substrates, according to established procedures (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545, 11, 25).

15

Western blotting and zymography. Confluent 100 mm dishes of fibroblasts, microvascular cells, and keratinocytes (each containing about 2 million cells) are treated with the appropriate peptides for 24 hours. Based on known procedures for Western blotting the media from cells treated with the other peptides, cells are treated with 10

20   μg/ml switch region prodomain peptide, serine–substituted peptide, or scrambled peptide per 20,000 cells. After treatment, cells are rinsed with PBS and lysed in ice-cold buffer (50 mM Tris-CI pH7.5, 150 mM NaCI, 2mM EGTA, 1% Triton X-100) containing protease inhibitors (1x complete protease inhibitor cocktail, Roche). The lysate is collected and centrifuged at 12,000 x g for 10 min at 4°C and the resulting pellet

25   resuspended in SDS-sample buffer (2% SDS (v/v), 62.5 mM Tris-HCI pH 6.8, 10% glycerol) with protease inhibitors and boiled at 100°C for 5 min and centrifuged at 12,000 x g for 10 min. The protein concentration in the resulting supernatant is determined by BCA microassay, using albumin standards also prepared in solubolization buffer. Prior to electrophoresis, samples are brought to 5% (v/v) 2-mercaptoethanol and incubated at

30   100^{0}C for 5min. Approximately 30 μg of total protein per sample in SDS buffer is resolved on 10% polyacrylamine gels, and the separated proteins transferred onto

polyvinylidene difluoride membranes (PVDF, Milllipore, Bedford, MA). Membranes are blocked for 1 hour in 0.1% (v/v) Tween-20 in PBS (PBS-T) containing 5% (w/v) nonfat dry milk prior to antibody binding. Membranes are then incubated for 1 hour at room temperature with antibodies at a dilution of approximately 1:5000 in blocking solution.

5   Then, membranes are incubated for 1 hour with goat anti-mouse IgG antibody conjugated to horseradish peroxidase (Jackson Immunoresearch Labs, West Grove, PA) at a dilution of 1:5000 in blocking solution. The membranes are processed for detection by enhanced chemiluminescence (ECL reagent, Amersham, Arlington Heights, IL). The amount of cellular MMP in each band will be quantified using Quantity One software (Bio-Rad,

10 Hercules, CA) by comparing to the corresponding recombinant MMP standard curve generated from the same blot. For zymography, adherent cells and their media is assayed three times, according to standard procedures (Livant *et al.*, (2000) Cancer Res. 60: 309-320).

15 . **Example 4**

Testing the effects of the MMP 1 cysteine switch peptide on dermal wound healing in obese diabetic mice and in their heterozygous, non–diabetic littermates

Biopsy punches 4 mm in diameter (Henry Schein, Inc., Port Washington NY) are used to wound C57Bl6 KsJ *db/db* and C57Bl6KsJ *db/+* mice as previously described (Livant *et al.*,

20 (2000) J. Clin. Invest. 105: 1537-1545). *Db/db* mice and their non–diabetic, *db/+* littermates are obtained at 6 to 8 weeks of age from Jackson Laboratories (Bar Harbor ME).

Db/db mice are aged at least an additional 2 weeks prior to use in wound healing experiments, to make certain that they display the impaired wound healing phenotype characteristic of this strain. Treatment groups consist of 10 to 20 dermal wounds each, or 5 to

25 10 *db/db* or *db/+* mice with duplicate dermal wounds. Anesthetized mice are wounded once or twice on the upper back by pinching the skin away from the underlying fascia and muscle, and pushing the biopsy punch through the skin as it lies over a forefinger. When wounded without penetrating the fascia or underlying muscle, bleeding is not apparent in dermal wounds in *db/db* and *db/+* mice (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545). Thus,

30 added peptide is not expected to be significantly diluted as it is delivered to wounds. As previously judged by measuring sections of day–old wounds with a reticle at 400–fold

magnification (Livant et al., (2000) J. Clin. Invest. 105: 1537-1545), *db/db* mice are expected to be wounded to a mean depth of approximately1.7 mm by this procedure. The depth of the wounds in the *db/+* mice is expected to be somewhat less because of the reduced amount of subcutaneous fat in these animals. However, in no case is underlying muscle wounded. In

5   each experiment, the mice are age–matched. Based on preliminary dose response studies with the MMP–7 switch region propeptide, approximately 10 µg of the Ac-PRCGVPDVAQF-NH$_2$ peptide from MMP–1 proprotein switch region is added to wounds in a volume of 5 µl normal saline (NS). The corresponding peptide containing the C–to–S substitution at the third position, Ac-PR$\underline{S}$GVPDVAQF-NH$_2$ is also assessed for its ability to promote *db/db* wound

10   healing. To verify the lack of activity in the peptide containing the C–to–S substitution at the third position, a peptide consisting of the scrambled sequence from the MMP–1 proprotein switch region, Ac-VVDAPPFCGQR-NH$_2$ is also tested. To assess the locality of the effect of switch region propeptide treatment on dermal wound healing, some *db/db* and *db/+* mice receive peptide in only 1 of 2 duplicate wounds; while the remaining wound on each mouse

15   receive 5 µl of NS only. This strategy has been previously used to demonstrate the locality of PHSRN–mediated wound healing enhancement in *db/db* mice (Livant et al., (2000) J. Clin. Invest. 105: 1537-1545).

Example 5

20   **Histological analysis of wound reepithelialization and provisional matrix formation**

On day 0, 27 aged *db/db* mice or *db/+* mice are wounded in duplicate with a 4 mm biopsy punch as described above. Mice are divided into 3 treatment groups of 8 mice. The wounds of each group are treated once with 5 µl NS containing either 10 µg Ac-PRCGVPDVAQF-NH$_2$ (from MMP–1), Ac-PR$\underline{S}$GVPDVAQF-NH$_2$ (the corresponding C–to–

25   S mutant sequence, or NS. On days 1 through 9, a mouse from each treatment group is sacrificed. Its wounds are dissected out, each surrounded by unwounded skin. Thus, all the wounded mice are assayed in this experiment. Wounds are fixed for at least 3 days in 5% formaldehyde in PBS, and embedded in paraffin.

Prior to sectioning and staining with hematoxylin and eosin according to standard

30   procedures (Schnaper et al., (1993) J. Cell. Physiol. 156(2): 235-246), the blocks are cut to expose the wounded tissue near the center of each wound. For each wound, the

distance migrated from the wound edges by keratinocytes is measured in 5 groups of 3 serial 5-micron sections. Fifty sections, or 250 microns separate each group of 3 serial sections from the next group. The distance migrated is measured using a reticle and a standard grid with phase contrast optics at 100–fold and at 250–fold magnification. The entire wound bed or new dermal tissue is also examined in each section of each wound at 200 and 400–fold magnification. Macrophages in the provisional matrix (as recognized by their eosinophilic cytoplasm, large, euchromatic nuclei, and ruffled cell membrane) are counted, and the presence of activated fibroblasts (as demonstrated by their lightly staining, euchromatic nuclei and extensive, basophilic cytoplasm) is scored. The presence of thick type I collagen fibers in the provisional matrix, characteristic of contracting wounds, is also evaluated for each treatment group. These histological assays are performed as previously described (Livant *et al.*, (2000) J. Clin. Invest. 105: 1537-1545).

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention that are obvious to those skilled in the relevant fields are intended to be within the scope of the following claims.